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(54) Title: CONTROL OF POST HARVEST GENE EXPRESSION IN PLANTS BY THE USE OF HARVEST-REGULATED GENE PROMOTERS

(57) Abstract

The asparagine synthetase (AS) gene promoter from asparagus is identified. This promoter is used to direct post-harvested gene expression in plants or plant parts.

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CONTROL OF POST HARVEST GENE EXPRESSION IN PLANTS BY THE USE OF HARVEST-REGULATED GENE PROMOTERS

FIELD OF INVENTION

The invention relates to the use of an asparagines synthetase (AS) promoter in the control of postharvest gene expression in plants. The invention in particular relates to the isolation and characterisation of the asparagus AS promoter and its use in postharvest gene expression.

BACKGROUND

Postharvest Quality

Maintenance of postharvest quality places great demands on highly perishable horticultural and agricultural produce. Spoilage after harvest can result in the waste of 5-50% of the global production of horticultural commodities (Kader, 1992). Losses occur due to poor storage and handling conditions, development of bacterial and fungal infections as well as the natural deterioration of harvested crops prior to processing or consumption as fresh produce. Current technologies to extend the postharvest life of horticultural crops include appropriate temperature and humidity control, the use of controlled atmospheres (altered carbon dioxide and oxygen composition), films for packaging, the use of heat treatments. For forage crops, methods to extend the storage quality include drying and low humidity storage.

As well as preventing huge losses of available food through the development of appropriate postharvest storage technologies, the improvement of the aesthetic quality of horticultural produce has also become an important research focus. Development of new cultivars with altered colour, flavour and nutritional qualities have been continually achieved through the use of traditional breeding. With the advent of more targeted genetic engineering technologies fruit and vegetables with altered cell wall metabolism (Sheehy et al., 1988; Smith et al., 1988; Tieman et al., 1992; Tieman and Handa, 1994), ethylene status (Ayub et al., 1996; Wilkinson et al., 1997; Henzi et al., 1999), and colour (Bird et al., 1991) have been developed. Once the biochemical processes that determine the type and extent of postharvest

deterioration have been identified there is huge potential to apply genetic engineering approaches to intervene in these processes and reduce the rate of crop losses.

New crops though gene technology

To create new plants with altered (enhanced) postharvest qualities by genetic engineering requires a knowledge of the effects of the gene to be introduced or down-regulated, coupled with the most appropriate gene promoter to control the expression of the gene.

A gene promoter can be defined as the complete sequence of nucleic acids immediately preceding the ATG start sequence of the gene of interest, and which contains regulatory elements controlling the endogenous expression of the gene.

Gene promoters control gene transcription through the binding or removal of transcription factors to positive and negative regulatory elements within the promoter sequence. The interaction of the regulatory elements with the transcription factors denote the level of control - when, where and how much – of gene expression. The biochemical or physical environment of the plant cell is translated, via a signal transduction cascade, to the active form of a transcription factor within the nucleus which then binds to, or is removed from, a regulatory element within the promoter. This connects a situation experienced by the cell to a response in the nucleus with the outcomes being a change in gene expression, changed protein synthesis and finally a response in the cell effected by the protein.

Gene promoters used in plasmid constructs for introducing a new gene into a transgenic plant can be of several types – constitutive, temporal or spatial. The use of constitutive promoters e.g., 35S from cauliflower mosaic virus (Gardner et al., 1981), result in continuous gene expression at high levels throughout the plant. Temporal promoters induce gene expression at certain times, often in response to specific conditions e.g., Cu inducible promoters (Mett et al., 1996). Spatially induced promoters direct expression in specific plant organs e.g., the tuber-specific patatin type I promoter (Rocha-Sosa et al., 1989).

The production of specific quality traits in plants after harvest

If a constitutive promoter is used to down-regulate the expression of a gene that is involved in deleterious processes after harvest the gene is down-regulated in all parts of the plant during growth and development as well as after harvest. However, the expression of such genes may be necessary for the normal functioning of the plant prior to harvest and if this is the case the use of a constitutive gene promoter is inappropriate. Similarly, the introduction of a new quality parameter after harvest, such as a new colour or the ability to resist postharvest diseases may interfere with normal plant development. The use of a gene promoter that can restrict expression to only after harvest would minimize the impact of new gene expression on plant growth and development.

Control of expression of asparagine synthetase in harvested asparagus

Asparagus spears are rapidly growing, immature vegetative organs at harvest, and deteriorate quickly after harvest. Physiological changes such as reduced respiration rate, toughening and flavour changes are accompanied by biochemical changes (reviewed by Lipton [1990] and King et al., [1993]). Changes in gene expression also occur rapidly and in response to harvest (King and Davies, 1992; Davies and King, 1993). There are similarities between the harvest-related gene expression and that occurring during natural foliar senescence of the mature asparagus plant, suggesting that harvest induces premature senescence in the immature organ (King et al., 1995). The rapid rate of induction of gene changes in asparagus after harvest suggests tight genetic control of the harvest-induced senescence.

One of the most notable in the suite of changes in gene expression in asparagus after harvest is the upregulation of AS, particularly in the spear tips. The up-regulation of AS in spear tips begins 3 h after harvest (Davies and King, 1993) and follows a rapid depletion of soluble sugars, particularly sucrose, that begins within 2 h of harvest. Other highly perishable vegetables also respond to harvest by upregulation of AS gene expression. For example, AS transcripts are not detectable in broccoli florets at harvest but increase within 2 h of harvest, and continue to increase up to 24 h. In broccoli leaves, AS transcripts are not detectable at harvest, but increase as excised leaves turn yellow (Downs and Somerfield, 1997).

Glutamine-dependant AS catalyses the transfer of the amide group of glutamine to

aspartate, producing asparagine and glutamate. The transamidation reaction catalysed by AS facilitates the storage of nitrogen in the soluble, stable and carbon efficient transportable form of asparagine. The reaction catalysed by AS is particularly important during conditions of carbohydrate stress because asparagine has a lower C:N ratio than glutamine, making it the preferred nitrogen storage or transport molecule when carbon is limiting. For highly perishable vegetables, carbon (in the form of soluble sugars) quickly becomes limiting after harvest.

Sugars affect the expression of many genes involved in essential processes such as photosynthesis, glycolysis, glyoxylate metabolism, nitrogen metabolism, defence mechanisms and cell cycle regulation (Koch, 1996). High sugar levels repress the expression of genes involved in sugar production and induce expression of genes for utilisation and storage. Low sugars have the reverse affect. There appears to be multiple pathways for sugar sensing in plants, invoked by levels of either hexoses or sucrose (Halford et al., 1999). There is evidence that hexokinase acts as one sensor of carbohydrate levels within plant cells (Graham et al., 1994; Jang and Sheen, 1994; Davies et al., 1996) but the steps beyond the perception of the signal – including the interaction of down-stream transcription factors with regulatory elements on the gene promoter is, as yet, unclear (Jang et al., 1997). However, recent work has uncovered the nature of the sugar-responsive regulatory elements within two gene promoters. The promoter sequences for cucumber isocitrate lyase and malate synthase have been analysed in detail for information on the elements conferring a response to sugar-starved up-regulation. One of the sequence elements shared between cucumber isocitrate lyase and malate synthase promoters (IMH2), contains a core motif of CCCA which is involved in the sugar-induced down-regulation of malate synthase (Graham et al., 1994; Reynolds and Smith, 1995; Sarah et al., 1996).

That plant AS gene expression is regulated by sugar levels has only recently been demonstrated at the molecular level. In arabidopsis, three AS genes have been isolated, with one being up-regulated by sucrose and the others reciprocally down-regulated (Lam et al., 1994; Lam et al., 1998). In addition, excised maize root tips (Chevalier et al., 1996), and asparagus cell cultures (Davies et al., 1996) are negatively regulated by metabolisable sugar levels. The pea AS1 promoter activity is

reported to be repressed by sucrose (Ngai et al., 1997 [in reference to Ngai and Coruzzi, unpublished data]). In asparagus the rapid depletion of sucrose from spear tips is one of the earliest physiological changes detected after harvest.

Other plant AS promoters are sensitive to light/dark, with arabidopsis and pea AS promoters being negatively regulated by darkness (Tsai and Coruzzi, 1990; Tsai and Coruzzi, 1991; Lam et al., 1994; Neuhaus et al., 1997). However, a second AS gene in arabidopsis is induced by light (Lam et al., 1998) indicating different regulatory elements are present on this promoter. The regulation of the asparagus AS gene is independent of light levels (King and Davies, 1992).

OBJECT OF INVENTION

The object of the present invention is to provide the use of an AS promoter for senescence- and/or harvest-regulated gene expression in plants.

In particular, the object is to provide a promoter isolated from asparagus for use in harvest-regulated gene expression in plants or to at least provide a useful choice.

SUMMARY OF INVENTION

The invention provides an isolated plant gene promoter which is regulated by harvest and/or senescence of a plant a plant part.

Preferably the promoter is an asparagine synthetase (AS) promoter and is preferably isolated from asparagus, more preferably *Asparagus officinalis*.

In particular, the invention provides an asparagus AS promoter selected from the group comprising:

(a) the DNA sequence depicted in Figure 1;

(b) a fragment of the DNA sequence depicted in Figure 1 encoding a promoter which is regulated by harvest and/or senescence of the plant or plant part;

(c) a DNA sequence which is a degenerate equivalent of the sequence (a) or (b); and

(d) a DNA sequence hybridisable under standard conditions to a sequence (a), (b) or (c).

The invention also provides an isolated asparagus AS promoter. Preferably the AS promoter is isolated from *Asparagus officinalis*.

The invention also provides a suitable transformation vector or plasmid including the AS promoter.

The invention also provides the use of an AS promoter to control gene expression of a gene in a plant or plant part and preferably to control gene expression particularly in plant or a plant part after harvest and/or during senescence.

The invention also provides the use of the promoter to down-regulate gene expression in harvested plants or a plant part.

The invention also provides the use of the promoter in the identification of an element involved in harvest and senescence regulation.

The invention also provides the use of the promoter in the control of gene expression in response to changes in sugar concentration in a plant, or plant culture.

The invention describes the use of a gene promoter to control the expression of a gene related to postharvest quality in harvested horticultural and agricultural products.

The invention also relates to the use of the promoter to control the expression of new genes that allow the synthesis of new or novel substances or metabolites in plants or a plant part after excision.

The promoter described here is from *Asparagus officinalis* and directs the expression of the endogenous asparagine synthetase (AS) gene in asparagus spears in a harvest-responsive fashion. The isolated promoter is negatively affected by sugar levels, and is not responsive to light. This promoter can be used to direct the expression of introduced genes in either the sense or antisense orientation to improve the postharvest quality of horticultural products and to enhance the keeping quality of stored forage crops.

The invention also provides a transgenic plant containing the promoter according to the invention.

BRIEF DESCRIPTION OF DRAWINGS

The invention will now be described by way of example only and with reference to the accompanying drawings in which:

Figure 1 shows the promoter sequence of the asparagus AS gene.

Figure 2 is a schematic diagram of the asparagus AS gene.

Figure 3 is a photograph of transformed arabidopsis leaves histochemically stained

for GUS. A, AS-1 leaf after a 24 h postharvest treatment; B, AS-1 leaf at 0 h; C, 35S-GUS leaf at 0 h.

Figure 4 is a bar graph of GUS activity in transformed arabidopsis leaves using fluorometric detection.

Figure 5 is a graph of GUS activity in transformed arabidopsis leaves during growth in light/dark conditions, with or without 58 mM sucrose in the growing medium.

Figure 6 shows the transient analysis of GUS expression under the control of a 2 kb AS promoter fragment of the CAMV 35S promoter in asparagus calli. Asparagus calli incubated for 48 h on media + sucrose were shot with pRT99-GUS or pASP3. The calli were then incubated for a further 48 h on media + sucrose or – sucrose and then stained for GUS activity in the presence or absence of sucrose for 24 h.

KEY to Figure 6

	plasmid shot	**media before shooting**	**media after shooting**	**GUS assay buffer**
A	pRT99-GUS	- sucrose	- sucrose	- sucrose
B	pRT99-GUS	+ sucrose	+ sucrose	+ sucrose
C	pASP3	- sucrose	- sucrose	- sucrose
D	pASP3	+ sucrose	+ sucrose	+ sucrose
E	pASP3	+ sucrose	- sucrose	- sucrose
F	pASP3	- sucrose	+ sucrose	+ sucrose
G	pASP3	+ sucrose	+ sucrose	+ sucrose (24 h) then – sucrose (24 h)

Figure 7 is a northern blot of mRNA from 3 cm asparagus tips isolated from spears stored in air, or in the controlled atmospheres indicated for up to 6 days after harvest. The northern blot was probed with pTIP27 (a cDNA clone encoding asparagus asparagine synthetase).

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to the use of a gene promoter to control heterologous gene expression in harvested plants or plant parts. The gene promoter originates from asparagus and in this plant controls expression of AS in response to harvest. Given the features of the promoter, discovered through experiments relating to the harvest-

induced expression of the asparagus AS gene, and the direct analysis of the promoter (outlined in the following examples), this gene promoter can be used to control the expression of desirable genes in harvested (excised) plants or plant parts. This promoter can also be used to down-regulate gene expression in harvested plants or plant parts, using antisense or other down-regulatory techniques.

The promoter outlined here controls gene expression in plants after they are harvested (excised). The promoter is light-independent and can be repressed by high levels of sucrose. The promoter elements controlling sugar regulation appear to be separate from elements responsive to harvest, but may be invoked as conditions ensue in harvested plants or plant parts.

Harvest-regulated gene promoters can be used for (but are not limited to) the following:

- controlling the expression of genes introduced for the purpose of enhancing the postharvest quality of horticultural or agricultural crops (including fruit, vegetables, flowers, cereal and forage crops);
- controlling the expression of DNA sequences introduced for the purpose of suppressing the synthesis of undesirable proteins that lead to deterioration of postharvest quality of horticultural or agricultural crops (including fruit, vegetables, flowers, cereal and forage crops);
- controlling the expression of genes that confer insect or disease-resistance in harvested crops;
- controlling the expression of genes encoding enzymes that can breakdown harmful or undesirable substances in plants or plant parts after harvest (e.g., alkaloids in potatoes, tannins causing astringency);
- controlling the expression of genes for the purpose of *in planta* processing of secondary metabolites after harvest.

The examples below describe the sequence of the AS gene promoter, the induction of the promoter by harvest and repression by sucrose.

Plant material

Field grown *Asparagus officinalis* L. cv. Limbras 10 plants provided all spear material for DNA isolation.

Arabidopsis thaliana (L.) Heyn. (ecotype Columbia) was used in transformation experiments. Seed was surface-sterilised and germinated on half-strength Murashige and Skoog (MS) media (Murashige and Skoog, 1962). After the plants had reached 3 to 4 true leaves they were potted up into 5 cm pots covered with muslin and allowed to grow to maturity (approx. 4 weeks) in either a glasshouse or growth cabinet with a 16 h day photoperiod and a 15°C night/25°C minimum day temperature.

A. officinalis L. cv. Mary Washington calli gifted from Mr John Seelye (Crop & Food Research, Palmerston North, New Zealand) was used for analysis of promoter construct expression by particle gun transformation. Asparagus calli were maintained by incubation on MS basal medium with 3% (w/v) sucrose, 1 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2% Phytagel (Sigma). Asparagus calli were incubated under near dark lighting and 100% humidity at 22°C.

EXAMPLE 1

Construction of asparagus genomic library and isolation of asparagus AS genomic clones

Genomic asparagus DNA was partially digested with *Eco*RI restriction endonuclease (Boehringer Mannheim), ligated in EMBL4 phage arms (Stratagene) and packaged into phage particles using Gigapack II Gold packaging extracts (Stratagene) according to the manufacturers instructions. The library was titred at 1.2×10^6 pfu. The library was amplified and 8.5×10^4 pfu plated onto each of six 9 cm^2 LB plates. Duplicate plaque lifts from each plate using Hybond N^+ nylon membrane (Amersham) were fixed on 0.4 M NaOH-soaked filter paper. The membranes were prehybridised, hybridised to radiolabeled pTIP27 probe (asparagus cDNA clone encoding AS, Davies and King, 1993), and washed according to the methods described for Southern blot analysis above. Seven of the 27 positive signals identified on the primary screen were screened a further three times to obtain clonal populations of phage. DNA was isolated from each of the seven phage clones according to the methods of Sambrook et al. (1989). Restriction mapping and Southern analysis revealed six of the phage

clones contained the 16 kb *Eco*RI fragment of interest and one phage clone contained the 5.5 kb *Eco*RI fragment of interest identified by genomic Southern blot analysis. The 5.5 kb and 16 kb phage inserts were gel-purified from the EMBL4 phage arms using a DNA purification kit (BioRad). The 5.5 kb *Eco*RI fragment was subcloned into phosphatase-treated, *Eco*RI-digested pUC19 plasmid to produce plasmid pR200. Similarly, the 16 kb *Eco*RI fragment was subcloned into phosphatase-treated, *Eco*RI-digested pUC19 plasmid to produce plasmid pR100.

EXAMPLE 2

Sequence analysis of the asparagus AS promoter

Sequence analysis of nested deletion clones generated from pR200 and various subclones of pR200 and pR100 revealed the full length asparagus AS gene had been isolated (Moyle et al., 1996). Furthermore, pR200 was identified as containing approximately 3 kb of promoter sequence. Nested deletion of pR200 using a ds nested deletion kit (Pharmacia) resulted in the isolation of subclones providing sequence for one strand of the AS promoter. A 3 kb *Eco*RI/*Sac*I fragment from pR200 was directionally subcloned into *Eco*RI/*Sac*I-digested pBLUESCRIPT KS$^+$ to produce plasmid pR700. Nested deletion of pR700 resulted in the isolation of subclones providing sequence for the second strand of the AS promoter. All plasmids were prepared for sequencing using the Wizard DNA purification system kits (Promega) or QIAprep spin plasmid kits (Qiagen). All sequencing was performed using an automated DNA sequencer (Applied Biosystems), at the Otago Centre for Gene Research, University of Otago, Dunedin, New Zealand.

EXAMPLE 3

Construction of plasmids containing the AS promoter and GUS

An existing promoter-GUS reporter gene expression plasmid was altered by removing the existing promoter sequence and replacing it with approximately 2 kb of AS promoter from the 5.5 kb genomic clone isolated earlier. Two bases around the ATG translation start site of the asparagus AS gene were changed by site-directed mutagenesis using the Sculptor *in vitro* mutagenesis kit (Amersham) to form an *Nco*I site.

The AS promoter/GUS/CaMV poly-A expression cassette was cloned into the binary

plasmid pART-27 (Gleave et al., 1992) for *Agrobacterium*-mediated transformation of arabidopsis. This plasmid contains the nptII gene (conferring kanamycin resistance) as a selectable marker. This plasmid was called pAS-GUS. The binary vector alone (pART27) and the same vector bearing an intron-containing GUS gene under the control of the CaMV 35S promoter (pART27-10) were used as controls. All three plasmids were electroporated into the LBA4404 strain of *Agrobacterium tumefaciens* (Life Technologies).

The AS promoter/GUS/CaMV poly-A expression cassette was subcloned into the direct transformation plasmid pRT99 (Topfer et al., 1988) to produce a plasmid pASP3 used for transient expression analysis in asparagus callus cultures. A second plasmid, pRT99-GUS, was constructed containing a CaMV 35S promoter/GUS/CaMV poly-A expression cassette and was used as a positive transformation control.

EXAMPLE 4

Transformation of arabidopsis with AS promoter-GUS construct

The transformation of arabidopsis with an AS promoter-GUS construct was achieved using the method of Bechtold et al. (1993) with minor modifications. The LBA4404 strain of *Agrobacterium tumefaciens* (Life Technologies), containing the binary vector pAS-GUS was used for transformation of arabidopsis. LBA4404 *Agrobacterium* were grown with streptomycin (50 mg L^{-1}) and kanamycin (100 mg L^{-1}) for 16 h at 28°C in LB medium (final density of 2.0 AU at 600 nm). After centrifugation, the bacterial pellet was resuspended in the infiltration medium as specified by Bechtold et al. (1993) to a final density of 0.8 AU at 600 nm. Individual plants that had the main stem excised 5 days earlier to encourage flower production in side branches were immersed upside down in 500 ml batches of *Agrobacterium* and a vacuum applied for 5 minutes followed by quick release. Plants were laid on their sides for 24 h and kept covered by plastic for 2 days to prevent dehydration. Four to six weeks after planting, approximately 20-50,000 T_1 were bulk-collected and used to screen for kanamycin-resistant seedlings transformed with the different binary plasmids. From the seeds screened, 8 pAS-GUS seedlings, 1 pART27 seedling and 7 pART27-10 seedlings were isolated and grown on and individual T_2 seed collected. This seed was also germinated on kanamycin selection plates and individual resistant plants grown on to be used in the GUS experiments and for isolation of genomic DNA for

Southern analysis. T_3 seed was then collected from these plants.

EXAMPLE 5

Postharvest expression of GUS in transformed arabidopsis

Mature leaves from each of the kanamycin resistant T_2 plants were excised and stored in a petri dish in the dark at 20°C for 24 h to mimic general postharvest conditions. A small amount of water was added to prevent desiccation. To assess whether harvest had induced GUS expression via the AS promoter, the activity of the GUS enzyme was assayed using the standard methods of Jefferson et al. (1986) followed by histochemical detection as detailed by Stomp (1992) or detection by fluorometry as detailed by Gallagher (1992). For the histochemical determination of GUS activity a 24 h-stored leaf and a leaf removed just prior to the assay being performed (0 h) were cut with a scalpel blade to aid entry of the histochemical GUS substrate 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) and incubated at 37°C overnight. Where the GUS enzyme is present the GUS substrate is cleaved to release a blue compound. For the fluorometric assay, leaves were ground in extraction buffer and the protein extract was then incubated along with the fluorometric GUS substrate (4-methylumbelliferyl-□-D-glucuronide) to determine GUS activity. The protein content of this extract was also measured using the BioRad Protein Assay Dye Reagent. GUS activity is defined as pmol MU (min.μg protein)$^{-1}$ where MU is 4-methylumbelliferone, the free fluorescent moiety produced by the action of the GUS enzyme on the fluorescent substrate.

EXAMPLE 6

Effect of light and sucrose on GUS expression in transgenic arabidopsis

Four-week old kanamycin-resistant transgenic arabidopsis plants containing pAS-GUS were grown in MS media and then transferred to conditions where the light and sucrose conditions were varied. Growth media was either with or without 58 mM sucrose and plants were grown either in the dark or the light for the duration of the experiment. The alteration in GUS expression in tissues was monitored by fluorescence (detailed above).

EXAMPLE 7

Transformation of asparagus callus for transient expression analysis

Asparagus callus pieces (ca 0.5 cm^3) were subcultured onto fresh MS medium with 3% (w/v) sucrose, 1 mg L^{-1} 2,4-D and 0.2% Phytagel or onto MS medium with 1.4% (w/v) mannitol, 1 mg L^{-1} 2,4-D and 0.2% Phytagel 48 h prior to particle bombardment.

Plasmid pRT99-GUS or pASP3 was prepared using a Wizard Maxiprep DNA purification kit (Promega). Gold particles (5 mg, 1 μm diameter; BioRad) were washed once in 500 μl ethanol and twice in 500 μl sterile distilled water by vortexing and centrifugation. The gold particles were then resuspended in 500 μl of sterile distilled water. pRT99-GUS or pASP3 plasmid (20 μl of 2 mg/ml plasmid) was added to a 100 μl aliquot of the gold slurry and mixed by vortexing. $CaCl_2$ (2.5 M, 50 μl) was added to the DNA/gold mix followed by 20 μl of 100 mM spermidine. The gold slurry was vortexed after the addition of each component. The gold slurry was placed at 4°C for 5 min after which time 180 μl of supernatent was discarded.

A particle accelerating gun, with the modification of using a Nalgene desiccator as the vacuum chamber instead of a steel box, was used to transform the asparagus calli. DNA-coated gold slurry (2 μl) of was loaded into the centre of a sterile filter unit (Millipore) which was screwed into the luer-lok fitting inside the top of the particle gun desiccator chamber. A media tub containing the piece of asparagus calli to be transformed was placed on the stage at the bottom of the particle gun chamber directly under the filter unit. A vacuum of 95 kPa (14 psi) was applied to the chamber of the particle gun. The prechamber was loaded with 500 kPa (60 psi) helium which was subsequently fired through the particle gun by detonating the timer set to 50 milliseconds. The vacuum on the particle gun chamber was slowly released and the transformed asparagus calli removed and incubated for 48 h under near dark lighting at 22°C and 100% humidity.

EXAMPLE 8

Sucrose regulation of gene expression in asparagus calli using a particle gun-mediated transient expression assay

Asparagus calli were initially grown on media containing 58 mM sucrose but were transferred to media containing no sucrose or 58 mM sucrose and incubated for 48 h prior to the initiation of the experiment. The calli were bombarded with gold

particles coated with plasmid pASP3 or pRT99-GUS and incubated for 48 h to allow transient expression under sucrose-fed or sucrose-starved conditions. Transformed asparagus callus pieces were then histochemically assayed for transient expression of the GUS reporter gene using X-Gluc as a substrate. Asparagus calli were incubated in X-Gluc assay buffer supplemented with either 50 mM sucrose or 50 mM mannitol for 24 h at 37°C.

EXAMPLE 9

Analysis of gene expression in harvested wild-type asparagus spears in response to controlled atmosphere storage

Controlled atmosphere storage at room temperature is a postharvest storage condition known to retain quality and high sucrose levels in asparagus spears (Hurst et al., 1997). To test whether AS gene expression is upregulated in harvested spears when sucrose was not limiting (and hence whether the AS promoter could drive gene expression under these conditions), freshly harvested 18 cm spears were stored at 20°C in either air or controlled atmospheres of 10% O_2/10% CO_2 or 1.5% O_2/10% CO_2, balanced with N_2. Following storage for up to 120 h, mRNA was isolated from 3 cm spear tip sections according to the method of King and Davies (1992). Northern blots of RNA were probed with the cDNA clone pTIP27 encoding asparagus AS.

RESULTS

Cloning and characterisation of the asparagus AS promoter sequence

The 5.5 kb and 16 kb *Eco*RI fragments identified by genomic Southern blot analysis were isolated by screening an EMBL4 library constructed with *Eco*RI partially digested asparagus genomic DNA using radiolabeled pTIP27 (asparagus cDNA encoding AS) as a probe. The 5.5 kb *Eco*RI fragment and 2 kb of the 16 kb *Eco*RI fragment were sequenced. The full length AS gene was contained within the 5.5 kb *Eco*RI fragment and the 16 kb *Eco*RI fragment (Moyle et al., 1996). The 5.5 kb *Eco*RI fragment also contained approximately 3 kb of AS promoter sequence.

The promoter sequence of the AS gene is shown up to the nucleotide 5' to the ATG translation start site in Figure 1. The putative TATA box is shown in bold and underlined. Two pairs of large repeat sequences are shown in bold type and italics.

A GA repeat 5' to the ATG translation start site is shown in bold type. The first base of the second sequence in the largest pair of repeated sequences is superscripted. Box sequences repeated within the asparagus AS promoter or homologous to sequences within other plant AS promoters are underlined. A CCCA motif is present in the underlined, italic sequence at −1289 to −1299 bp. The structure of the whole AS gene is shown in Figure 2.

Postharvest induction of gene expression in arabidopsis transformed with the asparagus AS promoter-GUS construct

Five plants were vacuum infiltrated with the *Agrobacterium* suspension containing the pAS-GUS binary construct. An equivalent number of plants were also treated with *Agrobacterium* containing either a negative (pART27) or positive (pART27-10) control binary plasmid.

Blue staining of leaves was observed where the leaves had been cut and damaged with the scalpel blade on all the 24 h postharvest treated leaves from the pAS-GUS experiment (data only shown for one plant, AS-1, Figure 3A). This staining was absent on leaves cut fresh from the plant (0 h) but damaged in the same way at the same time (data only shown for one plant, AS-1, Figure 3B). Leaves from the plant treated with *Agrobacterium* containing only the binary plasmid pART27 showed no staining either at 0 h or 24 h after excision (data not shown). However the plants putatively transformed with the intron-containing GUS gene under the 35S constitutive promoter (pART27-10) had blue leaves at 0 h and 24 h after excision (data shown for one plant, pART27-10-1 at 0 h, Figure 3C).

To get a more accurate and measurable estimation of GUS activity in these tissues a fluorometric assay was used. When soluble protein extracts were prepared from leaves of these putative transgenic plants and assayed for the presence of GUS protein the same result as the histochemical determination was obtained (Figure 4). Leaves from wild type, untransformed arabidopsis plants have no GUS activity at either 0 h or 24 h after excision. This is the same for the plant putatively transformed with just the binary plasmid pART27. Plants putatively containing the GUS gene fused to the strong constitutive promoter from CaMV 35S had high levels of expression at 0 h which increased approximately 2-3 times with the 24 h

treatment. Leaves from the eight plants putatively expressing the GUS gene under the control of the asparagus AS promoter had low levels of GUS activity at 0 h which dramatically increased after a 24 h postharvest treatment. This increased activity had a wide range of variability from an 8-fold increase (AS-3) to over a 100-fold increase (AS-5) with an average increase of 47-fold.

Effect of light and sucrose on gene expression in transgenic arabidopsis

There were high levels of GUS expression induced in plants grown in either the light or the dark without sucrose (Figure 5). When sucrose was introduced into the growing medium the levels of GUS expression dropped dramatically within the first day of treatment and remained at these repressed levels for the duration of the experiment. This repression was independent of the amount of light received by the plants. The promoter retains insensitivity to light conditions when introduced into transgenic plants in a manner similar to its light-independence in governing the endogenous expression of AS in harvested asparagus (King and Davies, 1992).

Sucrose regulation of gene expression in asparagus callus using a transient expression assay

Plasmid pASP3 containing a 2 kb AS promoter fragment-GUS reporter gene construct and the positive control plasmid pRT99-GUS containing a CaMV 35S promoter-GUS construct were analysed for sugar-regulated gene expression using a particle gun-mediated transformation system. Calli transformed with the positive control CaMV 35S-GUS construct produced blue zones corresponding to GUS activity regardless of the presence or absence of sucrose (Figure 6). Calli starved of sucrose for 48 h and transformed with the 2 kb AS promoter-GUS construct produced blue zones corresponding to GUS activity when assayed in the absence of sucrose. Calli incubated in the presence of sucrose and transformed with the 2 kb AS-GUS construct produced no blues zones of GUS activity when assayed in the presence of sucrose. The experiment was repeated three times with five replicates per treatment. The promoter response to repress GUS expression in sucrose-abundant conditions for transgenic asparagus calli is similar to that found for transgenic arabidopsis reported above.

AS gene expression in harvested spears in response to controlled atmosphere storage

AS expression in air-stored and all CA-treated asparagus was identical both in timing and intensity (Figure 7). The AS promoter induced AS expression in asparagus under high levels of sugar even though earlier work (Davies et al., 1996) and experiments described above demonstrated that the promoter can be repressed by sucrose. Clearly, signals other than sucrose levels are involved in the harvest induction of the asparagus AS promoter and the promoter can control expression independently of sucrose.

DISCUSSION

The isolation of the asparagus AS promoter has permitted the analysis of AS promoter regulation of gene expression by harvest as well as a direct investigation of the promoter response to sucrose and light. This is useful to clarify whether the harvest response is coincident with loss of sucrose in the harvested plant. In arabidopsis, several AS genes have been studied (Lam et al., 1994; Lam et al., 1998) but no promoter sequences have yet been published. In arabidopsis there appears to be a reciprocal relationship between at least two of the AS genes in terms of light and sucrose repression and induction (Lam et al., 1998). Pea AS gene expression is up-regulated in attached leaves during dark treatments and repressed during light treatments (Tsai, 1991; Tsai and Coruzzi, 1991), and regulatory elements involved in the light response have been identified (Ngai et al., 1997). The pea AS1 promoter has also been reported as being repressed by sucrose (Ngai et al., 1997, in reference to Ngai and Coruzzi, unpublished data), and contains a CCCA core motif (Neuhaus et al., 1997) that in the cucumber isocitrate lyase and malate synthase promoters is involved in the response element controlling sugar down-regulation (Graham et al., 1994; Reynolds and Smith, 1995; Sarah et al., 1996). Analysis of the asparagus AS promoter revealed a CCCA motif within a fragment at −1289 and −1299 bp (Figure 1). This motif may be involved in sugar regulation as with the promoters for isocitrate lyase and malate synthase.

We have used vacuum infiltration and *Agrobacterium* infection to introduce the asparagus AS promoter fused to the GUS reporter gene into arabidopsis. This demonstrates that the AS promoter controls expression at high levels even in heterologous plant systems, and that the gene regulatory proteins and signal transduction mechanisms are conserved between species. The promoter appears to

retain functionality, both in the harvest and sucrose responsiveness. The promoter was also introduced via a gene gun into asparagus callus tissue, and retained functionality with respect to sucrose sensitivity in transient expression assays.

Because highly perishable harvested vegetable crops such as asparagus – the source of the promoter of interest - lose soluble sugars rapidly after harvest it could be thought that the harvest response is, in fact, a response to sugar deprivation. However, in harvested spears that were stored under CA conditions to retain at-harvest sucrose levels in the spear tips, AS was still expressed even though our prior experience suggested the abundance of sucrose should have resulted in repression of AS expression. This implies that there is a sucrose-independent harvest response. This does not rule out the likelihood that sucrose-sensitivity cannot be invoked later in the postharvest storage period.

It is interesting to note that the harvest signal does not involve ethylene sensitivity. Asparagus spears do not produce ethylene and are not sensitive to supplied ethylene after harvest. This suggests that the asparagus AS promoter does not contain regulatory elements involved in ethylene signal transduction. However, arabidopsis is known to be sensitive to ethylene and to use ethylene to coordinate the onset of senescence (Grbić and Bleecker, 1995). In transgenic arabidopsis plants the asparagus AS promoter is still operative in detached leaves even though other aspects of excision-induced senescence may be occurring. This ability to control gene expression without influence by ethylene means that desirable ethylene-coordinated events (such as ripening) can take place while undesirable ethylene-coordinated gene expression can be specifically halted using antisense genes controlled by the asparagus AS promoter.

It is to be understood that the scope of the invention is not limited to the described embodiments and therefore that numerous variations and modifications may be made to these embodiments without departing from the scope of the invention as set out in this specification.

INDUSTRIAL APPLICABILITY

The invention provides an asparagus AS promoter and its use in the control of

postharvest gene expression in plants or plant parts. The use of the isolated promoter will assist in controlling spoilage after harvest in crops. The promoter may also be useful in the response to sucrose levels in a plant/plant parts after harvest.

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CLAIMS

1. An isolated plant gene promoter which is regulated by harvest and/or senescence of a plant or a plant part.
2. A gene promoter as claimed in claim 1 which is an asparagine synthetase (AS) promoter.
3. A gene promoter as claimed in claim 2 which is isolated from asparagus.
4. The AS promoter as claimed in claim 3 which is isolated from *Asparagus officinalis.*
5. The AS promoter as claimed in claim 4 which has the DNA sequence selected from the group comprising:
   (a) the DNA sequence depicted in Figure 1;
   (b) a fragment of the DNA sequence depicted in Figure 1 encoding a promoter which is regulated by harvest and/or senescence of the plant or plant part;
   (c) a DNA sequence which is a degenerate equivalent of the sequence (a) or (b); and
   (d) a DNA sequence hybridisable under standard conditions to a sequence (a), (b) or (c).
6. A transformation vector or plasmid including a gene promoter as claimed in any one of claims 1-5.
7. The use of the promoter as claimed in any one of claims 1-5 to control gene expression in plants or a part of a plant during senescence and/or after harvest.
8. The use of the promoter as claimed in any one of claims 1-5 to down-regulate gene expression in harvested plants or a part of a plant.
9. The use of the promoter as claimed in any one of claims 1-5 to identify an element involved in harvest and/or senescence regulation in a plant.
10. An isolated asparagus asparagine synthetase (AS) promoter.
11. An AS promoter according to claim 10 which is isolated from *Asparagus officinalis*.
12. An AS promoter according to claim 10 or claim 11 which has the DNA sequence selected from the group comprising:
    (a) the DNA sequence depicted in Figure 1;

(b) a fragment of the DNA sequence depicted in Figure 1 encoding a promoter which is regulated by harvest and/or senescence of the plant or plant part;

(c) a DNA sequence which is a degenerate equivalent of the sequence (a) or (b); and

(d) a DNA sequence hybridisable under standard conditions to a sequence (a), (b) or (c).

13. The use of an asparagine synthetase (AS) promoter isolated from asparagus as claimed in any one of claims 10-12 to control expression of a gene in a plant or a part of a plant in response to changes in sugar concentration in the plant or plant culture.

14. The use of a promoter as claimed in any one of claims 1-5 to control expression of a gene in a plant or a part of a plant in response to changes in sugar concentration in the plant or plant culture.

15. The use of a gene promoter as claimed in any one of claims 1-5 to control expression of a gene related to quality in harvested horticultural and/or agricultural products.

16. A transgenic plant containing the gene promoter as claimed in any one of claims 1-5.

17. A transgenic plant containing an asparagine synthethase (AS) gene promoter isolated from asparagus.

18. A transgenic plant according to claim 17 in which the promoter is isolated from *Asparagus officinalis*.

19. A transgenic plant as claimed in any one of claims 16-18 in which the promoter controls the expression of a gene introduced into the plant to enhance the post-harvest quality of horticultural and/or agricultural crops.

20. A transgenic plant according to any one of claims 16-18 in which the gene promoter controls the expression of a DNA sequence introduced into the plant to suppress the synthesis of an undesirable protein that leads to deterioration of post-harvest quality of horticultural and/or agricultural crops.

21. A transgenic plant according to any one of claims 16-18 in which the gene promoter controls the expression of a gene that confers pest or disease resistance in a harvested crop.

22. A transgenic plant as claimed in any one of claims 16-18 in which the gene

promoter controls the expression of a gene encoding an enzyme that can breakdown harmful or undesirable substances in plants or a plant part after harvest.

23. A transgenic plant as claimed in any one of claims 16-18 in which the gene promoter controls the expression of a gene for the purpose of *in planta* processing of secondary metabolites after harvest.

24. A method of transforming *Asparagus* calli with a vector or plasmid containing the promoter according to any one of claims 1-5.

25. A method of transforming a plant or plant part or plant embryo or seed with the transformation vector or plasmid of claim 6.

26. Plants transformed according to the method of claim 25.

-3006 gaattc
-3000 tagaccttttcttccatttaatttcttcagcattagctaaggcaagaagc
-2950 cggtctttcaaaaagcttttagactgatttttcgagggagat*gaaaaaga*
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-2700 tgatatgtctcaacatgaatcaactagaaatcgtactaaggcaaactgct
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-2250 aaacaagaaaaccctcgtatatataccatgtgcctcaaatggtagataac
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-1750 ctttggtttgtgagtgtttgtttcgagaaatttaaaatgtcgacgtagtt
-1700 gtaatctacaaaagcaaagcataactttgtttgtaattgaggttgcatct
-1650 tttggttcactattacagaaatgagtcacttgctcattgaattggtcagt
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-1200 *aatcgctgttaaaatataaactataacactttattcttatcataacttt*
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-1100 *aattcaaattccactaactacaaatttaaaacaaattttaaaaatttcga*
-1050 *tatagctgccagttatcggcctttaaaatggctcctctaagtctcaatgt*
-1000 *aacatttcttttgctttaatatataataacatgatttctcttgtaattcc*
-950 *cgctaagattctcactctaatacattcttaatgttaaaaagtagacatct*
-900 *aaatatctaataaaataataattttttagtattcaccatgcctatctattc*
-850 ctctatacttataagatgatcttaagttttaccattcatgctatagtaaa
-800 tactacagtgaattagagtagcattattattatgcaacatcgttatagca
-750 caaaataaaatactgtagtattttttaaaaatattattataatattcaaaa
-700 atttactgtaacctttaaaaacgttattgtgacaaaaataaaaaagaggg
-650 ccatgttttacacgatgtggaaagagatactgtaatattattgcacgatg
-600 tgcgcactaatattattatatgcaacgataatatcttcatccatccagga
-550 taacattgaatactcagacaagaatcagcatatcagacgaaatagaaact
-500 tatcataccaagaaagtggaaagagatatttaaaaaacaataaaaaaata
-450 aagagtataaacaattgagattccttcaatattccacgctcttatccaca
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-350 gttcgtccttggatagatgggcggggacacgtgtaccgctccaaccgcaa
-300 ctgaccggtccttgttcaccgctgcggtggggcccagctgagtcatacct
-250 cggtgtcgaacaggatagcgtcgttgatcctcggcttcctttttactattg
-200 gatcgtaccggcggggtgaatcgctgtccggcacacaaccgacacgtgtc
-150 ggttgtccctggacgtgaaggaccgaagccgcttgggtttgggcctataa
-100 atagggagttttgttgagttatgagagcatattaattctcacttccacta
-50 ataagtacctctgcttgaaccttttgagagagagagagagagagaggatc

FIGURE 1

Schematic of asparagus AS gene

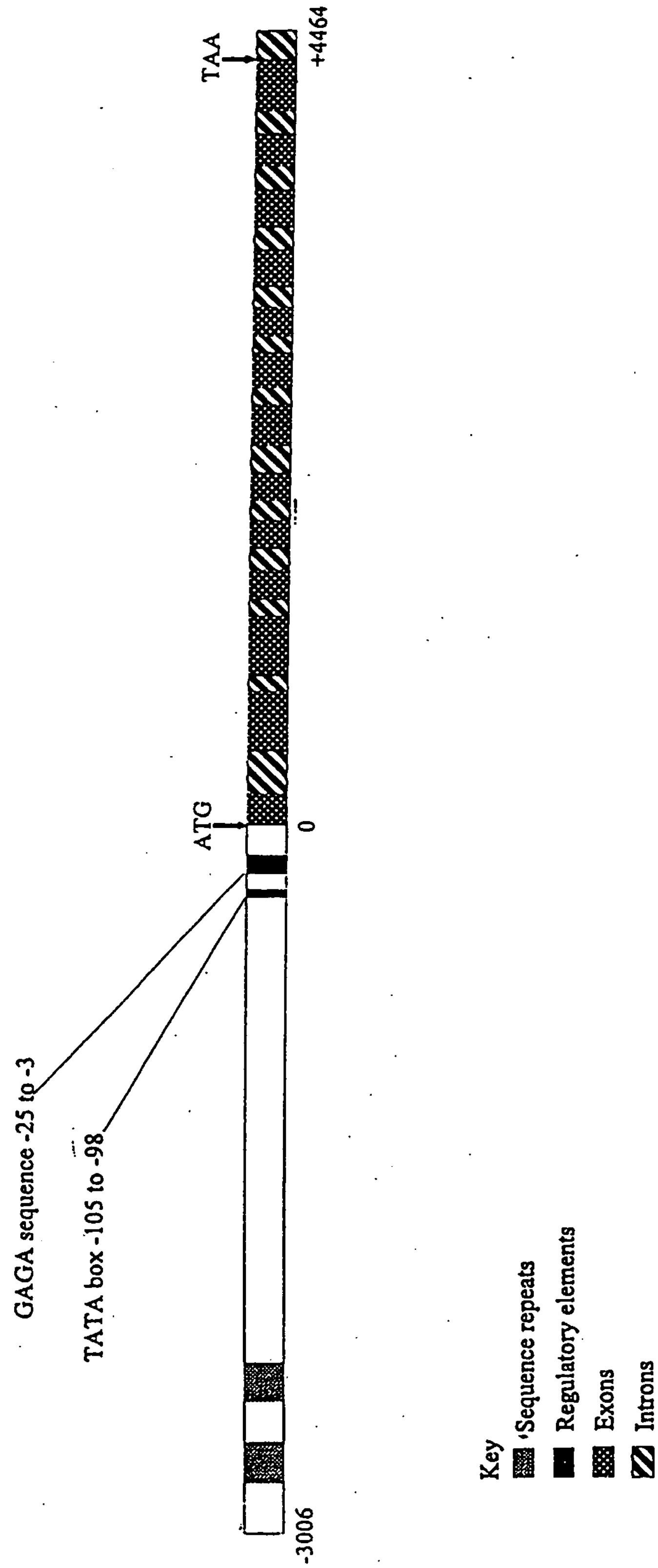

FIGURE 2

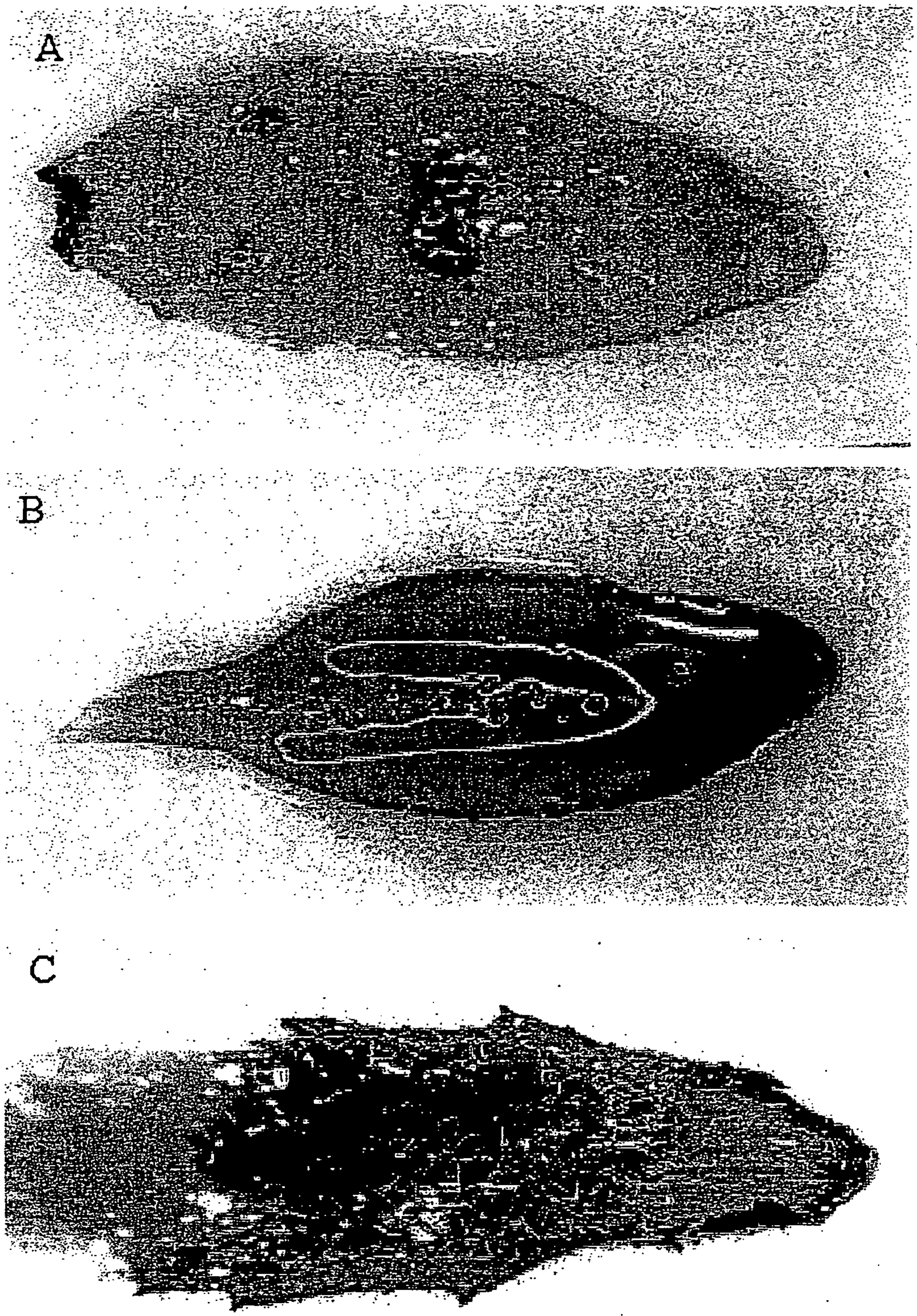

FIGURE 3

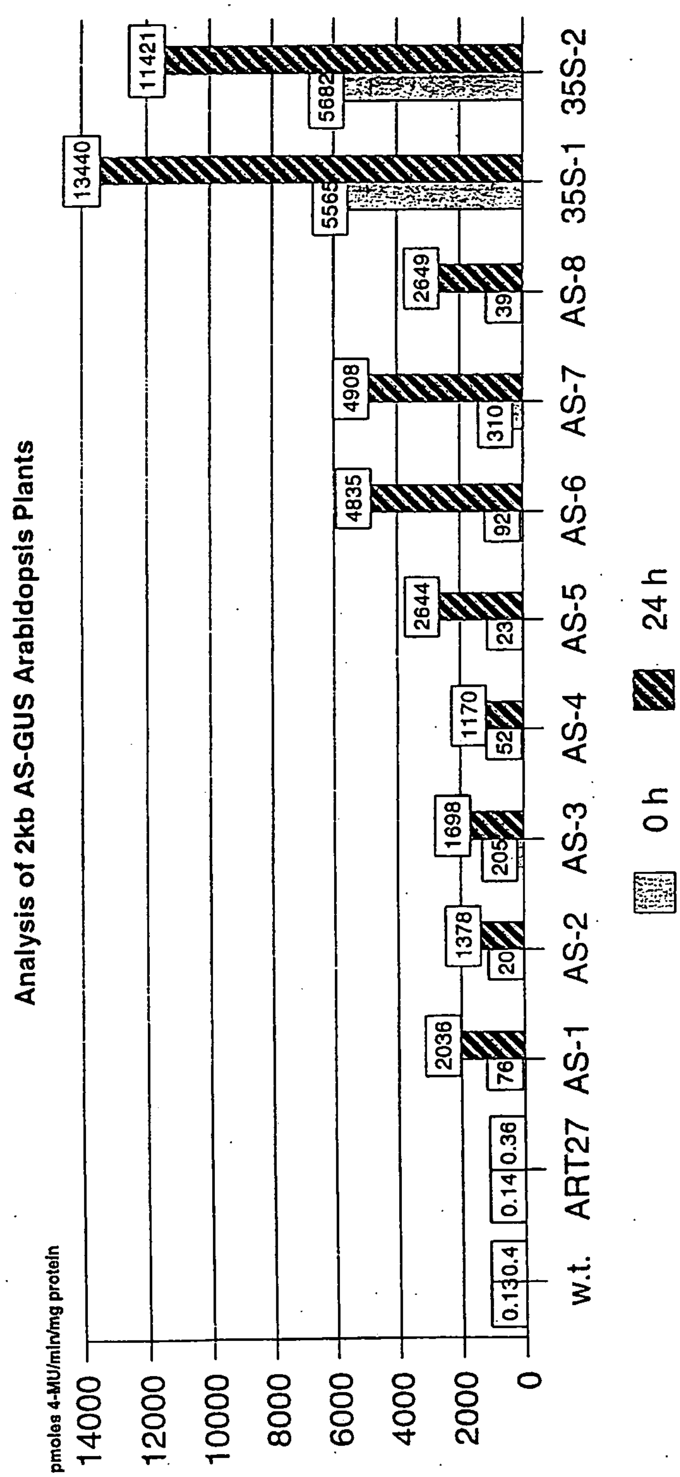
Analysis of 2kb AS-GUS Arabidopsis Plants
pmoles 4-MU/min/mg protein
14000
12000
10000
8000
6000
4000
2000
0
w.t.
ART27
AS-1
AS-2
AS-3
AS-4
AS-5
AS-6
AS-7
AS-8
35S-1
35S-2
0.13
0.4
0.14
0.36
76
2036
20
1378
205
1698
52
1170
23
2644
92
4835
310
4908
39
2649
5565
13440
5682
11421
0 h
24 h

FIGURE 4

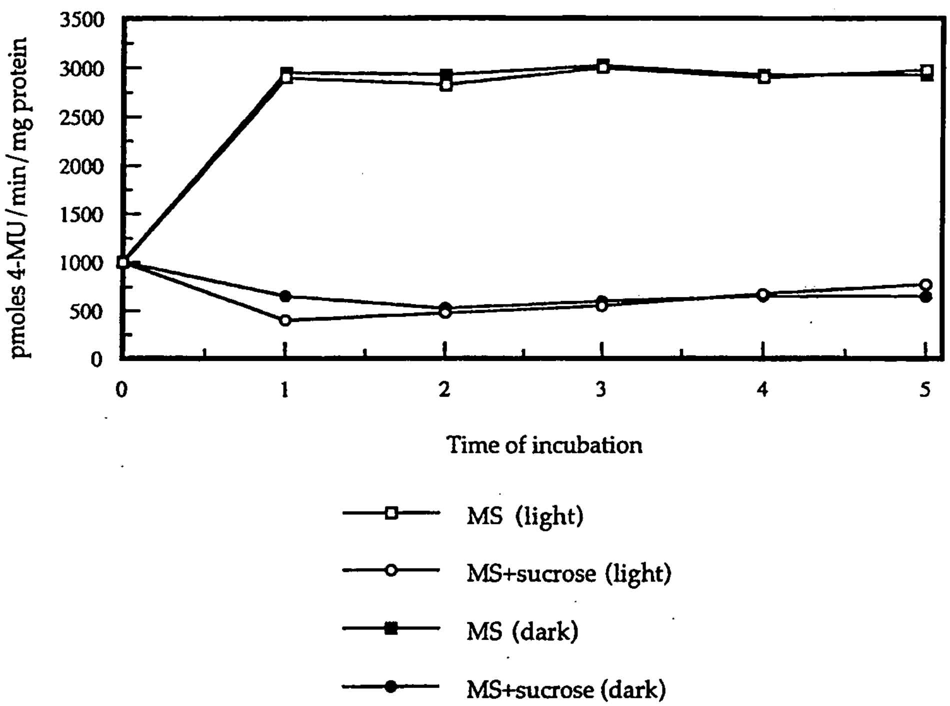
pmoles 4-MU/min/mg protein
3500
3000
2500
2000
1500
1000
500
0
0
1
2
3
4
5
Time of incubation
MS (light)
MS+sucrose (light)
MS (dark)
MS+sucrose (dark)

FIGURE 5

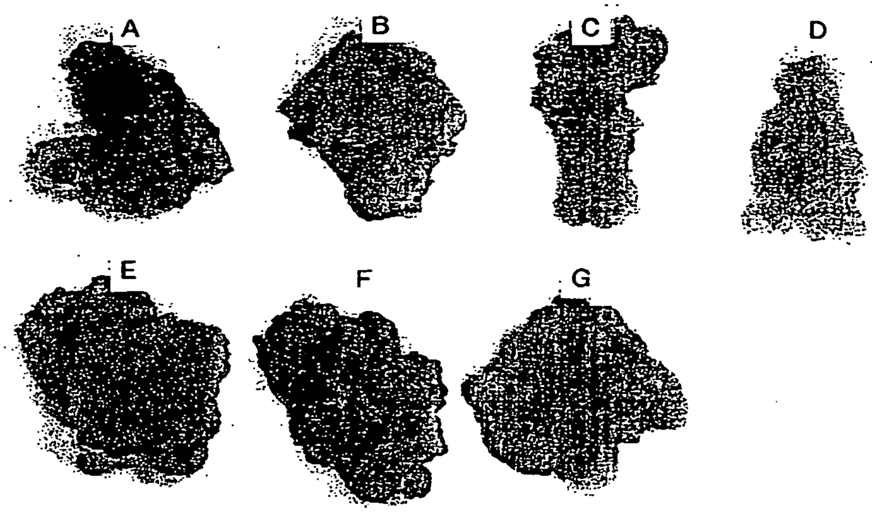

FIGURE 6

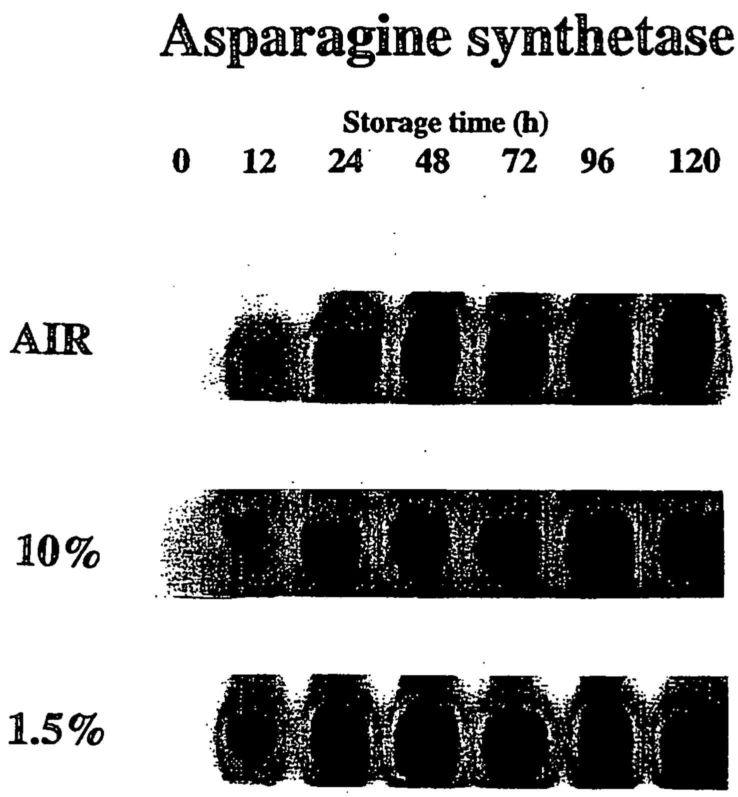
Asparagine synthetase
Storage time (h)
0 12 24 48 72 96 120
AIR
10%
1.5%

FIGURE 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ 99/00195

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[7]: C12N 15/11 A01H 1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
SEE ELECTRONIC DATA BASES

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CA, Medline, WPAT, Agricola, Biosis Preview
plants/CT AND ((asparagine()synethetase OR aspartate()ammonia()ligase)(s)promoter)
GenBank, EMBL, PDB Nucleic Acids: Figure 1

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	Naomi, O et al, *The Plant Cell*, 11 (1999) pp1073-1080. "Leaf senescence is delayed in tobacco plants expressing the maize homeobox gene *knotted1* under the control of a senescence activated promoter" Whole Document	1, 6-9, 16, 19-24
X	Ngai, N. *The Plant Journal*, 12(5) (1997) pp1021-1034 "Light-induced transcriptional repression of the pea AS1 gene: identification of cis-elements and transfactors" & EMBL Accession Numbers Y13321 and Y13322 Whole Document	1-26

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report 11 FEB 2000
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer **J.H. CHAN** Telephone No.: (02) 6283 2340

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ 99/00195

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,Y	Davies, K. M. & King, G. A. Plant Physiology 102 (1993) pp 1337-1340 "Isolation and characterisation of a cDNA clone for a harvest-induced asparagine synthetase from *Asparagus officinalis* L." Whole Document	1 - 24
X	Gan, S. & Amasino, R. M Science 270 (1995) pp1986-1988 "Inhibition of leaf senescence by autoregulated production of cytokinin" & EMBL Accession Number V37336 Whole Document	1, 6-9, 16, 19-24
X,Y	WO 90/13633 (THE TRUSTEES OF ROCKERFELLER UNIVERSITY) 15 November 1990 C12N 9/00 Page 13 lines 14 - 28 and Figures 12, 13; page 59 line 5 -page 62 line 5	1-24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ 99/00195

Box I **Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The scope of claim 1 is so broad that no meaning full search can be carried out. The claim includes any isolated promoter that is regulated by harvest or senescence of a plant whether or not it has been identified as such. It is not economically feasible to draft a search strategy that encompasses all promoters regulated by harvest or senescence. Consequently only the promoter, for Asparagine Synethetase, as disclosed in the specification has been searched.

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II **Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/NZ 99/00195

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	90/13633	AU	56451/90	US	5 595 896	US	5 955 651
		US	5 256 558				

END OF ANNEX